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DC-SIGN and L-SIGN Are High Affinity Binding Receptors for Hepatitis C Virus Glycoprotein E2*

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The hepatitis C virus (HCV) genome codes for highly mannosylated envelope proteins, which are naturally retained in the endoplasmic reticulum. We found that the HCV envelope glycoprotein E2 binds the dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) and the related liver endothelial cell lectin L-SIGN through high-mannose *N*-glycans. Competing ligands such as mannan and an antibody directed against the carbohydrate recognition domains (CRD) abrogated binding. While no E2 interaction with distant monomeric CRDs on biosensor chips could be detected, binding is observed if CRDs are closely seeded (K_d = 48 nM) and if the CRD is part of the oligomeric-soluble extracellular domain of DC-SIGN (K_d = 30 nM). The highest affinity is seen for plasma membrane-expressed DC-SIGN and L-SIGN (K_d = 3 and 6 nM, respectively). These results indicate that several high-mannose *N*-glycans in a structurally defined cluster on E2 bind to several subunits of the oligomeric lectin CRD. High affinity interaction of viral glycoproteins with oligomeric lectins might represent a strategy by which HCV targets to and concentrates in the liver and infects dendritic cells.

Hepatitis C virus (HCV)[1] is the major causative agent of non-A, non-B hepatitis throughout the world with more than 170 million people infected (1). Contamination with infected blood by injecting drug users is the primary risk factor for acquiring HCV infection. The majority of infected patients are unable to clear the virus, and many develop chronic liver disease, cirrhosis, and hepatocellular carcinoma (2). Replication of the HCV genome could be demonstrated *in vivo* and *in vitro* in liver hepatocytes (3, 4) and hematopoietic cells including dendritic cells and B cells (5, 6). However, the molecular mechanism by which the virus targets to these sites of replication, notably in the liver, is not known.

HCV is a small, enveloped, plus-strand RNA virus belonging to the family flaviviridae and genus *hepacivirus*. The HCV RNA genome is 9600 nucleotides in length and encodes a single polyprotein that is post-translationally cleaved into up to 10 polypeptides including three structural proteins (core, E1, and E2), located at the N terminus, and five nonstructural proteins (1, 7, 8). Shortly after translocation into the endoplasmic reticulum (ER), oligosaccharide transferase catalyzes addition of Glc3Man9GlcNAc2 complexes at up to 6 (E1) and 11 (E2) *N*-glycosylation sites (for review see Ref. 9). Glucose residues are removed by glucosidases I and II, and correctly folded proteins are released from ER chaperones calnexin and calreticulin (10–13). The transmembrane domains of E1 and E2 are responsible for both heterodimerization (14) and retention of the glycoproteins in a high-mannose EndoH-sensitive glycoform in the ER (15–17). By analogy to other flaviviruses it is assumed that HCV capsids bud from the cytoplasm into the ER and that enveloped particles follow the secretion pathway through the Golgi. However, attempts to produce secreted HCV particles *in vitro* have not been successful so far (18–20), and it is not known if E1 and E2 on mature infectious virions possess a high-mannose, complex, or mixed glycosylation.

Several receptors have been proposed that could play a role in HCV entry into hepatocytes. The low density lipoprotein (LDL) receptor has been shown to mediate HCV internalization via binding to virus-associated LDL particles (21, 22). A second putative HCV receptor, the tetraspanin CD81, has been identified as a high affinity binding receptor (1.8 nM) for soluble recombinant E2 from HCV genotype 1a (23, 24). CD81 and LDL receptor are expressed in most cell types and thus likely do not account for the hepatic tropism of the virus. Furthermore E2 binds to the hepatoblastoma cell line HepG2, which does not express CD81 (25). More recently two novel E2 binding receptors have been identified on HepG2 cells, the scavenger receptor type B class I (SR-BI) (25) and the galactose binding C-type lectin asialoglycoprotein receptor (26). The lack of an efficient cell culture model has precluded functional confirmation of these receptor candidates at the level of virus entry.

It has recently been shown that C-type mannose binding

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[1] The abbreviations used are: HCV, hepatitis C virus; ER, endoplasmic reticulum; LDL, low density lipoprotein; CRD, carbohydrate recognition domains; SFV, Semliki Forest virus; BHK, baby hamster kidney; sE2, soluble E2; TM, transmembrane; aa, amino acids; ECD, extracellular domain; EDC, *N*-ethyl-*N*′-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride; EndoH, endoglycosidase H; RU, resonance units; DC, dendritic cells; PNGase F; peptide:*N*-glycosidase F; mAb, monoclonal antibody; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; DMJ, 1-deoxymannojirimycin hydrochloride; FACS, fluorescence-activated cell sorter; NHS, *N*-hydroxysuccinimide; HIV, human immunodeficiency virus.

lectins DC-SIGN and L-SIGN (DC-SIGNR) can be used by viral and bacterial pathogens including HIV (27), Ebola virus (28), cytomegalovirus (29), and mycobacterium tuberculosis (30–32) to facilitate infection. Both lectins can act either in *cis*, by concentrating virus on target cells, or in *trans*, by transmission of bound virus to a target cell expressing appropriate entry receptors. DC-SIGN and L-SIGN are homotetrameric type II membrane proteins, which can bind mannose residues of viral glycoproteins through a C-terminal carbohydrate recognition domain (CRD) (33, 34). DC-SIGN is an adhesion receptor for ICAM-2 and ICAM-3 on T cells (35) and is expressed in dendritic cells, some subsets of macrophages, and placenta (36–38). The physiological role of L-SIGN is not known yet. High-mannose *N*-glycans have been shown to be important for attachment of HIV to DC-SIGN and L-SIGN (34, 39), and oligomerization of the extracellular domain was shown to be implicated in high affinity binding of glycopeptides (33).

In this report we show that DC-SIGN and L-SIGN are two novel HCV envelope binding receptors. High affinity interaction of E2 with both lectins depends on the presence of high-mannose *N*-glycans on the viral glycoprotein and oligomerization of the C-type lectin CRD. HCV interaction with L-SIGN might represent a strategy by which the virus targets to and concentrates in the liver. Indeed its localization on the endothelium lining hepatic sinusoids (40, 41) makes it an interesting candidate for the capture of enveloped hepatotropic viruses like HCV.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Human antibodies were produced as previously described (42), and mouse antibodies were a gift from J. Dubuisson. Human antibodies CBH2, CBH4B, CBH4G, CBH5, and CBH7 and mouse antibody H33 are directed against conformation-dependent epitopes, and H52 and CBH17 against linear epitopes. As control, human and mouse isotype control antibodies R04 and IgG2a were used. Phycoerythrin-conjugated mouse anti-DCSIGN FAB161B and mouse anti-LSIGN FAB162P were purchased from R&D Systems. 1B10 is a DC-SIGN mouse conformation antibody directed against the DC-SIGN CRD and was described previously (29). Anti-FLAG antibody M2 was purchased from Sigma. HIV gp140 was produced in Semliki Forest Virus (SFV) vector-infected baby hamster kidney (BHK) cells as described previously (43). Cloning of DC-SIGN cDNA was described previously (29) and DC-SIGNR (L-SIGN) cDNA was a gift from Dr. R. W. Doms, (University of Pennsylvania, Philadelphia, PA). HCV envelope-encoding plasmid pCAV711V was a gift of T. Miyamura, (National Institute of Infectious Diseases, Tokyo, Japan) (44).

Cell Lines and C-type Lectin Expression—HelaP4DC were generated by transduction with a DC-SIGN expressing lentiviral vector as previously described (45). Hek293T (2×10^7 cells) cells were transfected with 40 μg of DC-SIGN or L-SIGN expression plasmids using standard phosphate calcium transfection method as described previously (46). HeLaP4DC and Hek293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin. BHK cells were cultured in BHK-21 Glasgow's modified Eagle's medium with 5% FCS, 1% penicillin/streptomycin, 20 mM Hepes, and 10% tryptose phosphate broth. All products used for cell cultures were from Invitrogen.

Construction of Soluble E2 (sE2) Expression Plasmid—Plasmid pCAV711V was used as template to generate recombinant SFV coding for the soluble transmembrane (TM)-deleted form of E2. The sequence encoding for the ectodomain of HCV envelope protein (E2, amino acids (aa) 384–710) was amplified by PCR (Pwo DNA Polymerase, Roche Applied Science) using the sense primer 5′-ATATTGCGCGCATGTTC-ATGCCTTCTTCTTTT-3′ and the antisense primer 5′-TTTATATTAT-GCATTCACTTGTCATCGTCATCCTTGTAGTCGCCGACGACCGCCG-ACCCTATACC-3′. The antisense primer used for E2 codes for the FLAG tag (DYKDDDDK) peptide sequence. The amplified sequence was digested by *Bss*HII and *Nsi*I and introduced into respective sites of the pSFV2 vector (47–49). The recombinant plasmid pSFVsE2 was sequenced through the E2 coding region.

Expression and Purification of Recombinant Proteins—Recombinant SFVsE2 was produced by electroporation of *in vitro* synthesized vector RNA into BHK cells. Soluble proteins were produced by infection of BHK cells by SFVsE2 (multiplicity of infection 50) as previously described (43). At 6 h postinfection, medium was replaced by 0% FCS Glasgow's modified Eagle's medium for standard protein purification or for the production of radiolabeled sE2 by 0% FCS methionine/cysteine-free DMEM (ICN Biomedicals) prior to addition of 200 μCi·ml^{-1} [^{35}S]cysteine and methionine (Pro-Mix ^{35}S, Amersham Biosciences). Synthesis of sE2 was continued up to 24 h postinfection in the presence (sE2man) or absence (sE2cpx) of α-manosidase I and II inhibitors 1-deoxymannojirimycin hydrochloride (DMJ, 1 mM, Calbiochem) and swainsonine (5 mM, Sigma). Supernatants were clarified and concentrated in a 10-kDa concentration column (Biomax, Millipore) prior to purification on anti-FLAG monoclonal antibody M2 beads and elution by soluble FLAG peptide (DYKDDDDK, 150 ng·μl^{-1}) according to instructions of the supplier (Sigma). For surface plasmon resonance experimentations, a supplementary dialysis against phosphate-buffered saline (Biomérieux) was realized overnight at 4 °C using a Slide-A-Lyzer Cassette (Pierce) to eliminate free peptides and purification buffer.

The cDNA coding for the CRD (corresponding to amino acids 254–404) and for the extracellular domain (ECD) of DC-SIGN (corresponding to amino acids 66–404) of DC-SIGN were obtained by PCR, cloned into pET15b (Novagen) and verified by DNA sequencing. The plasmids were subsequently used to transform *Escherichia coli* C41(DE3), and proteins were expressed as inclusion bodies. Inclusion bodies preparation and refolding of the proteins have been done by dilution and dialysis as already described (33). Purification of refolded DC-SIGN CRD and DC-SIGN ECD were achieved by affinity chromatography in two steps (Ni^{2+} column equilibrated in 25 mM Tris-Cl, pH 7.8, 150 mM NaCl, and 4 mM $CaCl_2$ and eluted with a linear gradient of imidazole followed by a mannose-agarose column equilibrated in 25 mM Tris-Cl, pH 7.8, 150 mM NaCl, 4 mM $CaCl_2$, and eluted with the same buffer lacking $CaCl_2$ but supplemented in 10 mM EDTA as described (29)). Pooled fractions were then concentrated and dialyzed against 25 mM Tris, pH 7.8, 150 mM NaCl, and 4 mM $CaCl_2$.

Deglycosylation and Protein Analysis—Crude protein extracts were denatured in 0.5% SDS, 1% β-mercaptoethanol at 100 °C for 5 min, and followed by incubation overnight at 37 °C in G7 buffer (50 mM sodium phosphate, pH 7.5, Biolabs), Nonidet P-40 buffer (1% Nonidet P-40, Biolabs) containing endoglycosidase H (2 milliunits, Roche Applied Science), or peptide:*N*-glycosidase F (PNGase F) (1000 units, Biolabs). To obtain native deglycosylated sE2, the denaturing step was omitted from this procedure. Mock-treated sE2 was prepared without PNGase F as control in binding experiments. Proteins were analyzed by SDS-PAGE (Nupage Novex Bis-Tris Gels, Invitrogen). Gels were either fixed, dried and exposed to Biomax MR1 film (Kodak), or stained with Novex Colloidal Blue Staining Kit (Invitrogen) according to the supplier's instructions.

Immunodetection of DC-SIGN, L-SIGN, and sE2 Proteins—DC-SIGN and L-SIGN were detected by fluorescent-activated cell sorting (FACS) using, respectively, phycoerythrin-conjugated anti-DC-SIGN (FAB161B) and anti-L-SIGN (FAB162P) antibodies. Cells were washed in FCS-free DMEM medium and resuspended in FACS analysis buffer (1% bovine serum albumin, 0.2% γglobulin, 0.1% sodium azide (all from Sigma)) followed by incubation with primary antibodies at a 1/50-dilution for 30 min at 4 °C. Cells were washed and fixed with paraformaldehyde (3.2%) prior to FACS analysis (BD Biosciences and data processing with CellQuest software (Becton Dickinson). For Western blotting, proteins were harvested (48-h cells post-transfection for Hek293T-L-SIGN) in lysis buffer (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100) supplemented with protease inhibitors (Roche Applied Science). Cell lysates or sE2 solution was subjected to SDS-PAGE and transferred onto a Immobilon P membrane (Millipore). Incubation with primary antibodies 1B10 (anti-DC-SIGN; 2 μg·ml^{-1}), FAB162P (anti-L-SIGN; 1 μg·ml^{-1}), MAbM2 (anti-FLAG; 4 μg·ml^{-1}) was followed by incubation with an anti-mouse horseradish peroxidase-conjugated secondary antibody NA931V (Amersham Biosciences) (1/1000). Bound antibody was detected by exposure to enhanced chemiluminescence detection reagents (Supersignal, Pierce) and analyzed by a video acquisition system (Intelligent Dark Box II; Fuji) and Image Gauge software (Fuji).

For immunoprecipitation of radiolabeled sE2, crude protein extract was incubated with 60 μl of protein G-Sepharose (50% suspension in A buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA (Invitrogen), 0.2% Nonidet P-40 (Fluka)) and mouse or human monoclonal isotopic antibodies (15 μg·ml^{-1}) for 30 min at 4 °C. Different primary antibodies (15 μg·ml^{-1}) were added to supernatant and placed overnight under agitation at 4 °C. After a 5-s spin at 13,000 rpm, the pellets were washed three times with A buffer and then B buffer (10 mM Tris-HCl, pH 7.5, 500 mM NaCl, 2 mM EDTA, 0.2% Nonidet P-40) and C buffer (10

mM Tris-HCl, pH 7.5). Beads were then resuspended in Laemmli buffer and heated at 100 °C for 3 min, and samples were separated by SDS-PAGE. Subsequently, gels were fixed, dried, and exposed to Biomax MR1 film (Kodak).

Surface Plasmon Resonance-based DC-SIGN CRD and ECD Binding Assay—Flow cells of a Biacore B1 sensor chip were activated with a mixture of 0.2 M *N*-ethyl-*N*′-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride (EDC) and 0.05 M *N*-hydroxysuccinimide (NHS) as described (29). Flow cell one was then blocked with 50 μl of 1 M ethanolamine, pH 8.5, and served as a control surface. The other ones were treated with the CRD (5–50 $\mu g \cdot ml^{-1}$) or the ECD (2.5 $\mu g \cdot ml^{-1}$) of DC-SIGN, both in 10 mM acetate buffer, pH 4. Different amounts of immobilized protein were obtained by varying the injected volume (see "Results"). Remaining activated groups were blocked with 50 μl of 1 M ethanolamine, pH 8.5. For binding assays, either sE2man or sE2cpx were diluted in running buffer (25 mM Tris, 150 mM NaCl, 4 mM $CaCl_2$, pH 7.8) and injected (at 15 $\mu l \cdot min^{-1}$) over the DC-SIGN-activated sensorchip for 15 min, after which the formed complexes were washed with running buffer. Surfaces were regenerated by a 5-min pulse of 100 mM EDTA. In some cases, surface was made with sE2man. For that purpose, sE2man, 5 $\mu g \cdot ml^{-1}$ in 10 mM acetate buffer, pH 4, was injected over an EDC/NHS-activated B1 sensorchip. Binding assay was performed with the conditions described above, DC-SIGN being injected over the sE2man surface.

sE2 Binding on Plasma Membrane-expressed DC-SIGN and L-SIGN—Assays were performed on 3×10^5 DC-SIGN or L-SIGN expressing cells in 200 μl of FACS buffer supplemented with 1 mM $CaCl_2$ and 2 mM $MgCl_2$ and ^{35}S-labeled sE2 protein at various concentrations. sE2 was incubated for 2 h at 4 °C, and unbound radioactivity was removed by three washes with FACS buffer. Cell pellets were resuspended in FACS buffer prior to addition of optiphase supermix solution (Wallac), and bound activity was counted in a 1450 Microbeta Trilux γ counter (Wallac). For inhibition assays, cells were preincubated with inhibitors in FACS buffer for 15 min at 4 °C before addition of labeled envelope protein preparations containing inhibitor. Inhibitors were used at 20 $\mu g \cdot ml^{-1}$ final concentration (mannan (Sigma), EGTA (Sigma), 1B10, isotype control IgG, HIV gp140). The native deglycosylated sE2 protein was compared with a mock-treated sE2 protein (incubated in buffer without PNGase F). Saturation binding was assayed by the addition of increasing amounts of labeled sE2 protein. Data were analyzed using Prism software (GraphPad), and the specific binding curves were fit by nonlinear regression after subtracting nonspecific binding, *i.e.* sE2man binding to HeLa cells.

RESULTS

Expression and Purification of High-mannose and Complex E2 Glycoforms—The HCV envelope glycoproteins are retained in the ER in a high-mannose glycoform by the action of their TM domain. When produced as soluble proteins, deleted of the TM domain, the proteins acquire complex glycosylation in the Golgi. Whether E2 on HCV virions acquires complex glycosylation is not known at present. To study interaction of HCV E2 with cell surface receptors, we expressed the extracellular domain of E2 (sE2), corresponding to aa 384–710 of isolate NIHJ1 fused to a 9-aa FLAG peptide at the C terminus (Fig. 1). We used the defective SFV vector as it allows high level expression of functional virus glycoproteins (43, 50). To mimic the glycosylation pattern observed on ER-retained wild-type E2, we treated SFV-infected BHK cells with α-mannosidase inhibitors DMJ and Swainsonine. The resulting protein, sE2man (58 kDa) acquires only high-mannose *N*-glycans as evidenced by sensitivity to endoglycosidase H (EndoH) digestion, resulting in a 39-kDa protein (Fig. 2*A*). In contrast, sE2cpx synthesized in untreated cells acquired complex glycosylation (65 kDa) and was mainly EndoH-resistant (Fig. 2*A*). A small shift in molecular weight was observed for EndoH-treated sE2cpx suggesting that a small fraction of glycosylation sites remained in their high-mannose EndoH-sensitive form. However both sE2man and sE2cpx were sensitive to PNGase F digestion, which cleaves both complex and high-mannose *N*-glycans at the Asn-GlcNac bond, resulting in a deglycosylated 37-kDa protein. To evaluate the role of high-mannose *N*-glycans in attachment to cell surface molecules, we deglycosylated sE2man by PNGase F

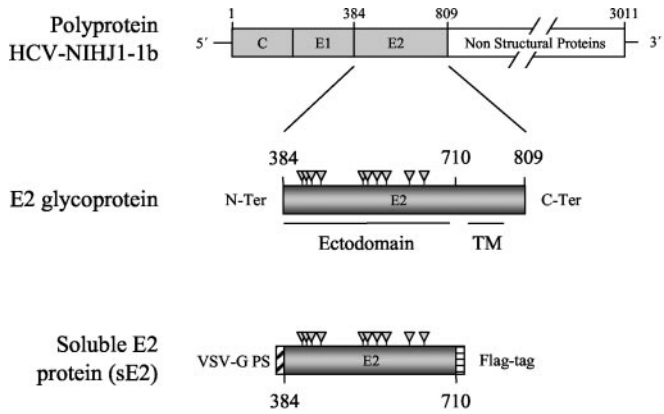

FIG. 1. **Generation of epitope-tagged soluble HCV glycoprotein E2.** The cDNA sequence corresponding to the ectodomain of E2 (aa 384–710; isolate NIHJ1) was inserted into the Semliki Forest virus expression vector. Transmembrane and C-terminal domains were replaced by the FLAG tag peptide. The VSV-G signal peptide (VSV-G PS) was added in the N-terminal domain of soluble E2 protein (sE2) (44). The *triangles* indicate the 10 putative glycosylation sites of $E2^{NIHJ1}$.

treatment under non-denaturing conditions (Fig. 2*A*, PNGase F native). sE2 synthesized in hepatocytic cell lines HepG2 and Huh7 had a complex EndoH-resistant glycosylation phenotype similar to sE2 produced in BHK cells (data not shown).

Soluble E2 Proteins Are Recognized by Conformation-dependent Monoclonal Antibodies—We analyzed whether ^{35}S-labeled sE2man and sE2cpx were correctly folded by immunoprecipitation analysis with a series of linear and conformation-dependent mouse and human monoclonal antibodies (Fig. 2*B*). All antibodies directed against conformation-dependent and linear epitopes, but not isotype control antibodies, recognized sE2man and sE2cpx. Correct folding of sE2 expressed in the SFV system is underscored by the lack of retention and degradation by the ER quality control machinery and efficient transition of sE2 to the Golgi (data not shown). For subsequent binding experiments sE2man and sE2cpx were purified by immunoaffinity using FLAG tag beads. Fig. 2*C* shows that sE2-enriched crude supernatants of SFV-infected cells can be purified by this system resulting in a greater than 90% pure sE2 preparation.

Soluble E2 Binds to DC-SIGN and L-SIGN—C-type mannose lectins DC-SIGN and L-SIGN were expressed on the surface of HeLa cells or HEK293T cells and could be detected by FACS and Western blot (Fig. 3*A*). DC-SIGN and L-SIGN monomers migrate at an apparent molecular mass of 45.5 and 48.5 kDa, respectively. DC-SIGN multimers could also be detected, in accordance with the capacity of both lectins to form dimers and tetramers (33). Both purified ^{35}S-labeled sE2cpx and sE2man were able to bind to DC-SIGN and L-SIGN but not to control cells (Fig. 3*B*). However at equimolar concentrations of radiolabeled sE2man and sE2cpx, an average of 10–20-fold higher binding signal was obtained for sE2man. This suggests that the presence of high-mannose, EndoH-sensitive glycosylation on sE2man facilitated binding. We further sought to identify the specificity of this interaction by testing several known inhibitors of ligand binding to DC-SIGN or L-SIGN. The lectin ligand mannan and the HIV envelope glycoprotein gp140 efficiently inhibited binding of sE2man and sE2cpx to both lectins (Fig. 4, *A* and *B*). Deglycosylation of sE2man greatly reduced binding of E2 to both lectins. In addition, the CRD-specific antibody 1B10 inhibited binding of sE2man and sE2cpx to DC-SIGN. Altogether these results identify DC-SIGN and L-SIGN as novel receptor molecules for HCV binding and suggest a pivotal role of high-mannose *N*-glycans as binding motifs on HCV E2.

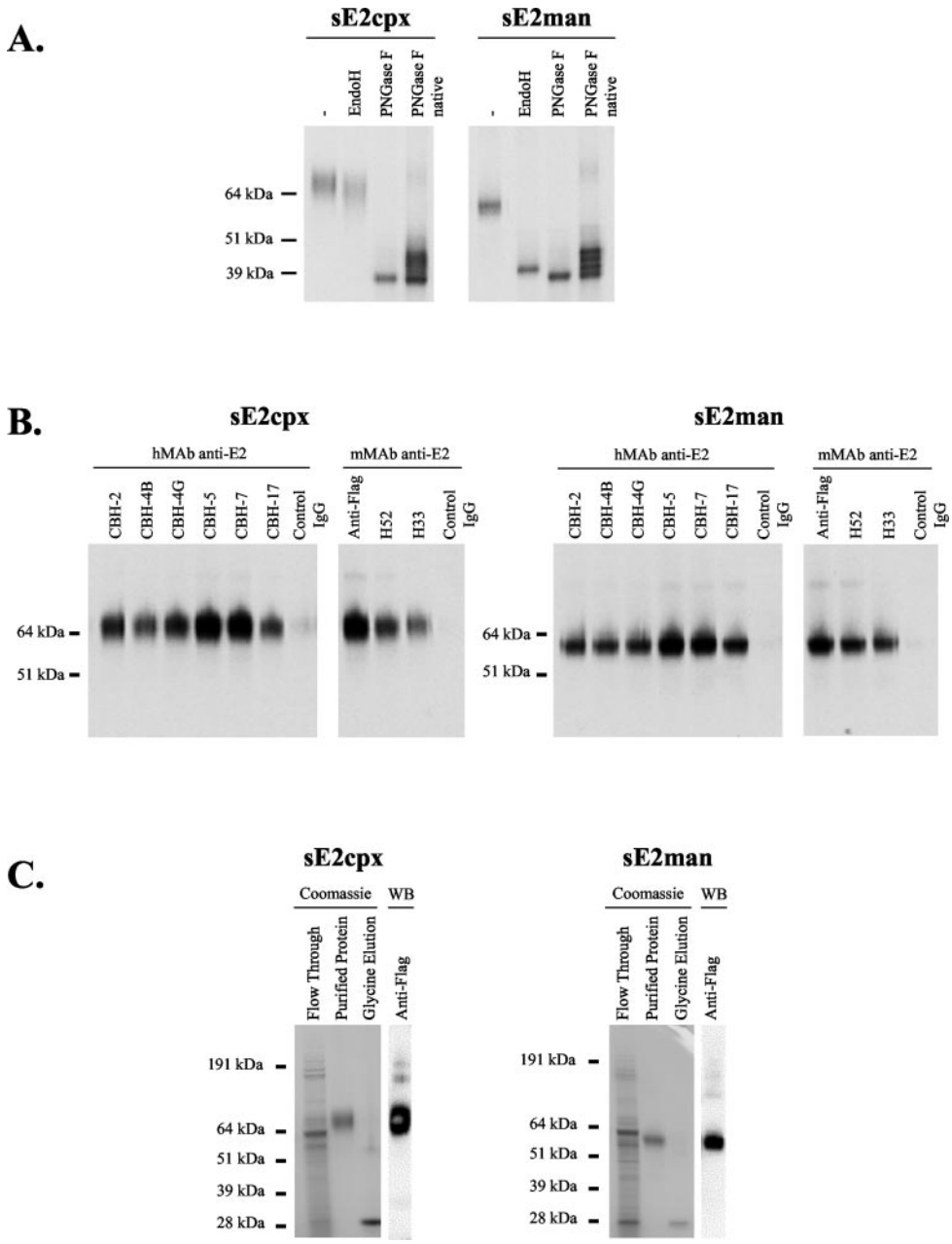

FIG. 2. **sE2 can be produced and purified with high-mannose or predominantly complex glycosylation.** *A*, ^{35}S-labeled sE2 was produced in BHK cells in the presence or absence of mannosidase inhibitors (1 mM DMJ and 5 mM Swainsonine) (sE2man or sE2cpx, respectively). Secreted proteins were subjected to digestion with EndoH or PNGase F and immunoprecipitated with an anti-FLAG epitope monoclonal antibody. sE2 could be partially deglycosylated with PNGase F under non-denaturing conditions (PNGase F native). *B*, antigenicity of sE2cpx and sE2man. Human mAb (*CBH*) and mouse mAb (*H*) directed against E2 conformation-dependent (*CBH2*, *CBH4B*, *CBH4G*, *CBH5*, *CBH7*, *H33*) and linear epitopes (*CBH17*, *H52*, *anti-FLAG*) were used to immunoprecipitate non-denatured ^{35}S-labeled sE2 protein. *C*, purification of sE2cpx and sE2man. Both sE2 proteins were purified under non-denaturing conditions by immunoaffinity using FLAG tag-Sepharose beads and elution with a FLAG peptide. The purity of sE2 was greater than 90% as evaluated by Coomassie Blue staining, and the specificity was controlled by Western blotting using the mouse mAb anti-FLAG.

sE2 Interaction with Monomeric and Oligomeric CRD of DC-SIGN—Surface plasmon resonance analysis (Biacore) was used to further investigate the E2/DC-SIGN complex formation (Fig. 5). In a first approach, the ECD of DC-SIGN was immobilized on the sensorchip to a level of 700 RU, and sE2 was injected in the fluid phase. Fig. 5*A* shows the binding curves when a range of concentration (see the figure legends) of either sE2man or sE2cpx was injected over the surface. Visual inspection of the binding curves showed that while sE2man injection over the surface gave rise to typical sensorgrams, sE2cpx did not show significant binding activity. Injection of identical concentrations of sE2man or sE2cpx over a control surface that underwent exactly the same chemistry (EDC/NHS activation, ethanolamine blocking), except from functionalization with DC-SIGN, did not produced any binding signal (data not shown). The formed complexes were fully dissociated with EDTA, indicating that ligand binding presumably involved an interaction between mannose residues on the viral glycoprotein and the CRD of DC-SIGN in a Ca^{2+}-dependent manner, as expected for this C-type lectin. However, when sE2man or sE2cpx were injected over a surface made with 200 RU of the CRD (Fig. 5*B*, "low density" surface) no binding was observed. This result suggested that binding can only be achieved when several CRDs are clustered in close proximity as it occurs in the

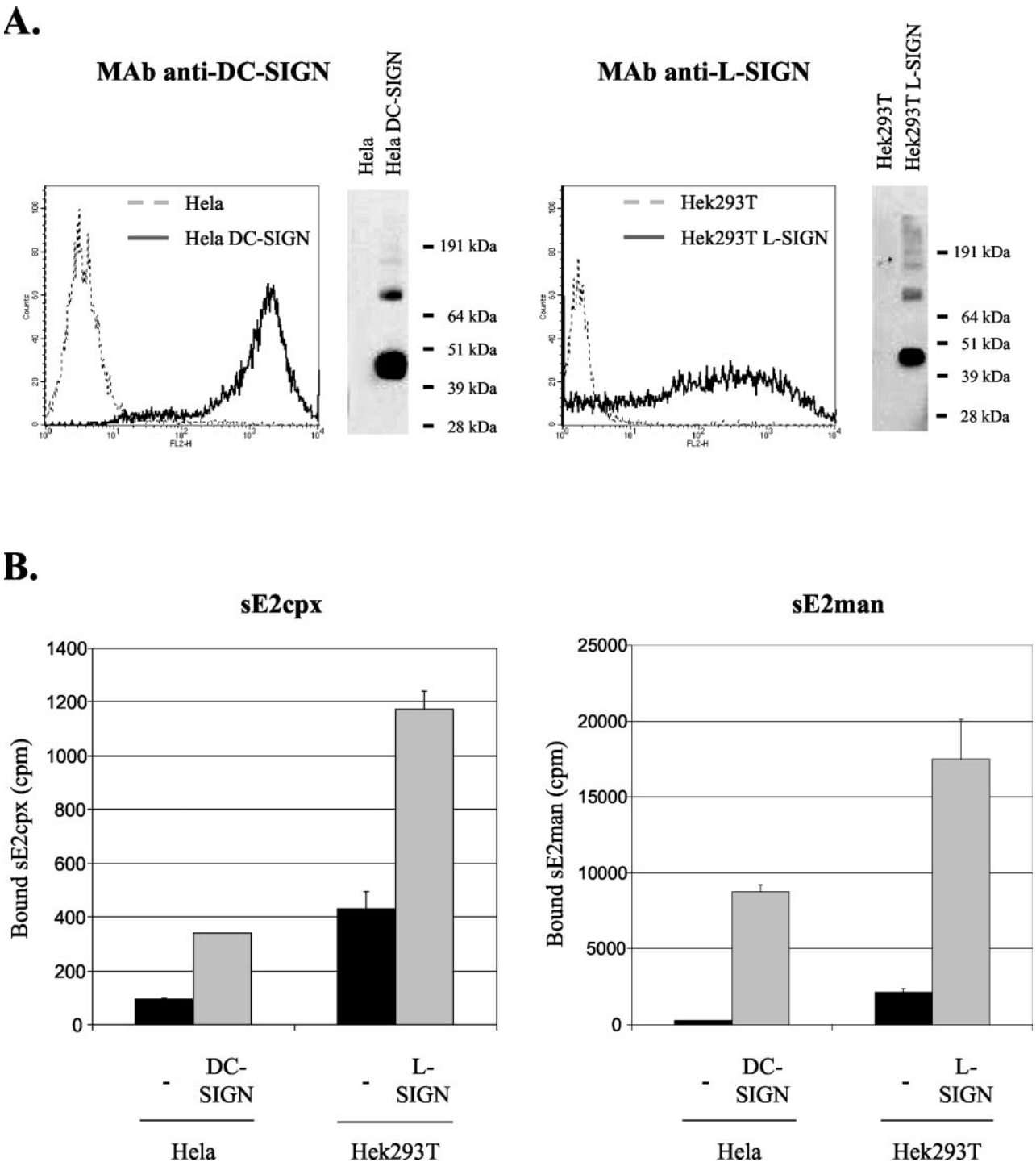

FIG. 3. **HCV E2 binds to C-type lectins DC-SIGN and L-SIGN.** *A*, expression of DC-SIGN in the HeLa P4DC cell line and L-SIGN in HEK293T cells was detected by FACS and Western blotting under non-reducing conditions. *B*, ^{35}S-labeled sE2cpx and sE2man were bound to HelaP4DC (4 nM sE2 added to cells) and HEK293T-L-SIGN (25 nM sE2 added to cells). The data are representative of three independent experiments. Values are given as the mean of duplicates ± S.E.

full-length DC-SIGN ECD. To investigate this possibility the binding of sE2man to DC-SIGN CRD was analyzed using a surface made with 1000 RU of the CRD ("high density" surface). Such a surface displayed high binding capacity (Fig. 5*C*), suggesting that binding is only achieved by the clustering of several CRD. The binding curves obtained have been fitted to various kinetic models. However, none of them could be satisfyingly fitted to any of the experimental data. This suggests a complex binding mode, a point consistent with the view that multiple CRDs cooperate to bind their ligand. Thus, where possible, equilibrium data were extracted from the sensorgram at the end of each injection and used to calculate the equilibrium dissociation constant independently of the kinetic analysis. This analysis returned a dissociation constant of 48 nM (Fig. 5*D*) and 30 nM (Fig. 5*E*) for the binding of sE2man to the ECD and the CRD high density, respectively. To further support the fact that clusters of CRD are required to interact with sE2, the CRD or the full-length ECD (which displays four clustered CRD) was injected over a surface made up with sE2man. As expected from the above hypothesis, the ECD efficiently bound to sE2man, while the isolated CRD produced virtually no binding response.

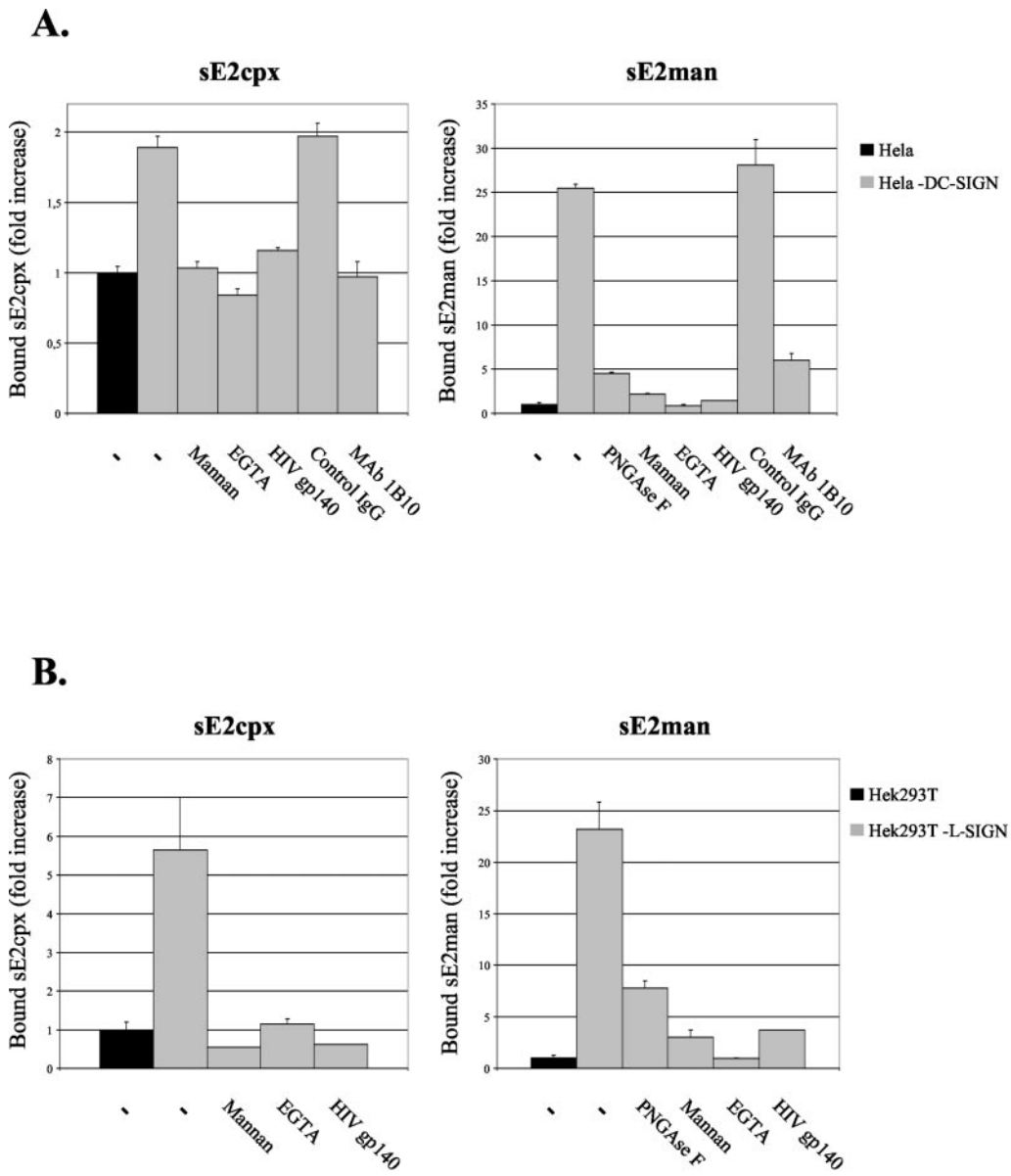

FIG. 4. **Specificity of sE2cpx and sE2man binding to DC-SIGN and L-SIGN.** Several known inhibitors and competitors for C-type lectin binding were used in a binding assay with ^{35}S-labeled sE2cpx and sE2man. *A*, specificity of binding to DC-SIGN. sE2cpx (20 nM) or sE2man (3 nM) were incubated on HelaP4DC cells in the presence of mannan (20 $\mu g \cdot ml^{-1}$), EGTA (5 mM), HIV gp140 (150 nM), or mAb 1B10 directed against the CRD of DC-SIGN (20 $\mu g \cdot ml^{-1}$) and a control IgG. Values are given as fold increase over background. Deglycosylation of sE2man (PNGase F native) was used to control that binding was mediated by E2 *N*-glycans. Mean cpm values for negative control HeLa cells were 32 cpm (sE2cpx) and 82 cpm (sE2man). *B*, specificity of sE2cpx and sE2man binding to L-SIGN. sE2cpx (20 nM) or sE2man (3 nM) were incubated in the presence of mannan (20 $\mu g \cdot ml^{-1}$), EGTA (5 mM), HIV gp140 (150 nM). Deglycosylation of sE2man (PNGase F native) was used to control that binding was mediated by E2 *N*-glycans. Mean cpm values for negative control HEK293T cells were 77 cpm (sE2cpx) and 141 cpm (sE2man). The data are representative of three independent experiments. Values are given as the mean of duplicates ± S.E.

sE2man Binds with High Affinity to Oligomeric Plasma Membrane-expressed DC-SIGN and L-SIGN—The dissociation constant of sE2man binding to DC-SIGN and L-SIGN was determined on lectin-expressing cells (Fig. 6). Saturation experiments revealed a dissociation constant of 3 nM for DC-SIGN (Fig. 6*A*) and 6 nM for L-SIGN (Fig. 6*B*). At 10–20 nM concentrations, when sE2man had reached saturation binding, the sE2cpx binding curve was still linear (data not shown), indicating that the dissociation constant of sE2cpx is higher than that of sE2man. However, we were unable to produce sufficient quantities of purified sE2cpx to obtain saturation of all binding sites on DC-SIGN- or L-SIGN-expressing cells. The low binding of sE2cpx is likely due to binding of single high-mannose *N*-glycans on sE2cpx to the lectin CRD, while sE2man might interact with the tetrameric CRD via several high-mannose *N*-glycans. Altogether binding results obtained with sE2man and sE2cpx indicate that multiple high-mannose *N*-glycans on HCV E2 are required to engage in a high affinity complex with tetrameric CRD of C-type lectins DC-SIGN and L-SIGN.

DISCUSSION

HCV glycoprotein E2 is highly mannosylated and retained in the ER through its TM domain (15–17). Previous studies indicate that TM-deleted E2 alone can assume a correct folding conferring binding to receptor candidate molecules CD81 (23) and SR-BI (25) and conformation-dependent antibodies (42). During synthesis in the ER, only correctly folded glycoproteins are released from molecular chaperones BIP, calnexin, or calreticulin before proceeding to the Golgi compartment (10, 12).

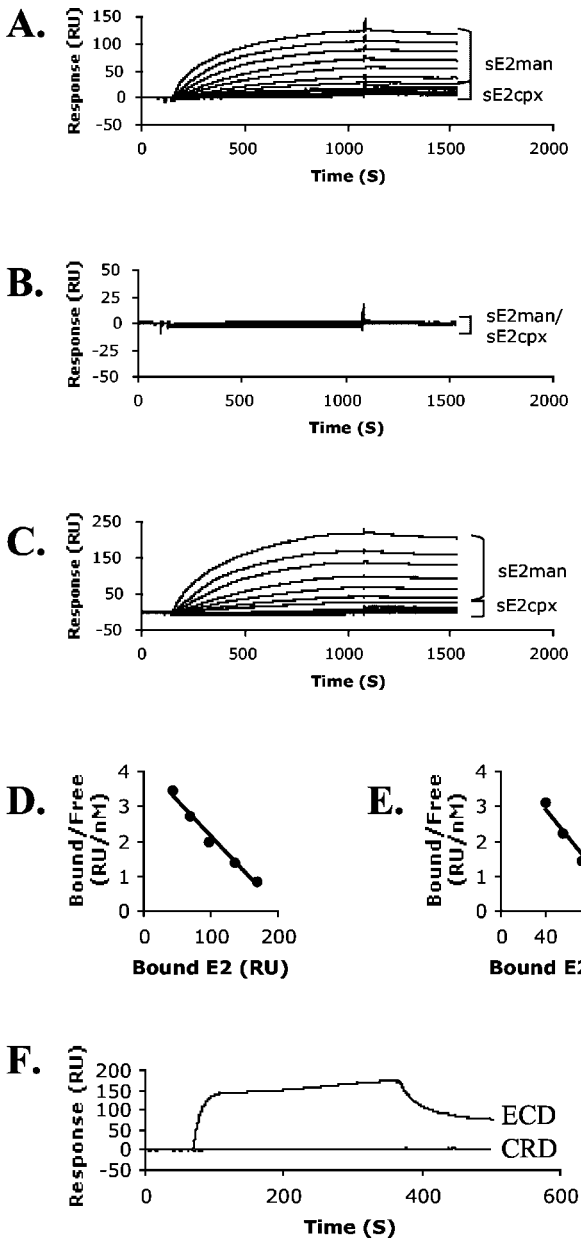

FIG. 5. **Surface plasmon resonance analysis of sE2/DC-SIGN interactions.** The sE2 glycoproteins, used at concentrations of 400, 200, 100, 50, 25, 12.5, and 6.25 nM, were injected for 15 min over DC-SIGN-activated surfaces at a flow rate of 15 $\mu l \cdot min^{-1}$, after which running buffer alone was injected. *A*, overlay of sensorgrams showing binding of either sE2man or sE2cpx to ECD surface. *B*, overlay of sensorgrams showing binding of sE2 (either sE2man or sE2cpx) to low density CRD surface (200 RU immobilized). *C*, overlay of sensorgrams showing binding of either sE2man or sE2cpx to high density CRD surface (1000 RU immobilized). *D*, Scatchard plot of the equilibrium binding data measured at the end of the sensorgrams for the CRD high density-sE2man interaction. *E*, Scatchard plot of the equilibrium binding data measured at the end of the sensorgrams for the ECD-sE2man interaction. *F*, overlay of sensorgrams showing binding of either the ECD or the CRD of DC-SIGN (both at 250 nM) to immobilized 120 RU of sE2man. Results are given as the mean of K_d values determined from two independent experiments.

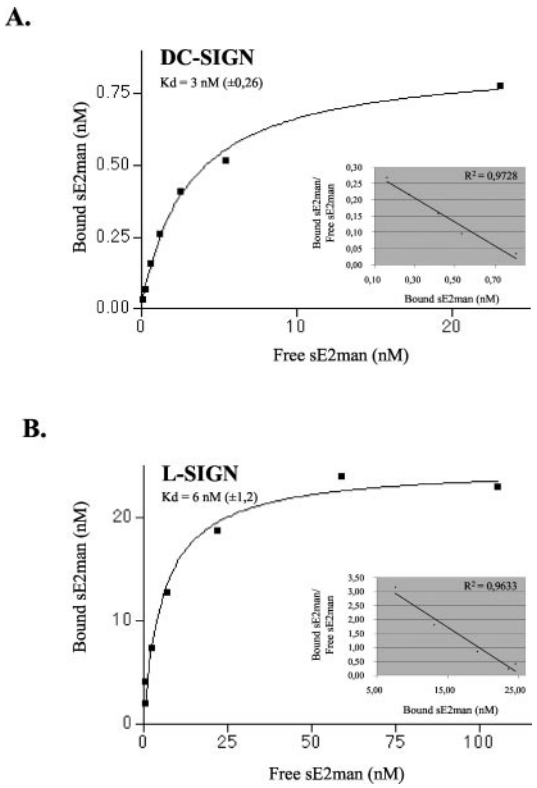

FIG. 6. **Saturation binding of sE2man on DC-/L-SIGN.** *A*, increasing amounts of ^{35}S-radiolabled sE2man protein were added to HeLa cell line constitutively expressing DC-SIGN. The saturation binding curve was fit by nonlinear regression after subtracting nonspecific binding, *i.e.* binding of sE2man to HeLa cells, from total binding to HeLa-DC-SIGN cells. *B*, increasing amounts of ^{35}S-radiolabled sE2man protein was added to Hek293T cells transiently transfected with an L-SIGN-expressing plasmid. The saturation binding curve shown was fit by nonlinear regression after subtracting nonspecific binding, *i.e.* binding of sE2man to control cells, from total binding to L-SIGN. The binding curves shown in *A* and *B* are representative of two independent experiments each. K_d values (±S.E.) were calculated using GraphPad Prism Software, and Scatchard plots of the equilibrium binding data are shown in the *insets*.

Proteins that are not correctly folded are retained by the quality control machinery in the ER and eventually become degraded. Time course experiments show that sE2man and sE2cpx are efficiently secreted from SFV-infected BHK cells (data not shown). No accumulation of intracellular aggregates is observed and after 6 h postentry into the ER, ≥90% of sE2 has migrated to the Golgi compartment as judged by the acquisition of complex glycosylation for sE2cpx. Furthermore, secreted sE2man and sE2cpx are recognized by a panel of conformation-dependent human monoclonal antibodies (42), including MAbCBH2 and MAbCBH5, which are able to block virus binding to CD81 (42). Interestingly, it was recently described that MAbCBH2 exclusively reacts with E2 when coexpressed with E1, implying that MAbCBH2 recognizes an E2 heterodimer-specific epitope (51). We do not confirm this finding as E2 is recognized by MAbCBH2 in the absence of E1 expression suggesting that the formation of the CBH2 epitope does not strictly depend on E1 coexpression and heterodimerization. However, it has to be noted that the E2 protein used in this study (genotype 1b; isolate NIHJ1) (44, 52, 53) is different from those used by Cocquerel *et al.* (51) and that recognition by MAbCHB2 could be isolate-specific.

We used TM-deleted soluble E2 that has either high-mannose EndoH-sensitive *N*-glycans (sE2man) or mixed, predominantly EndoH-resistant *N*-glycans (sE2cpx). Our results show that C-type lectins DC-SIGN and L-SIGN are two novel HCV envelope binding receptors, which bind E2 with high affinity (K_d = 3 and 6 nM, respectively). Like HCV E2, HIV gp120 has been described to bind with high affinity to DC-SIGN (K_d = 1.5 nM) (36). sE2cpx has only few EndoH sensitive high-mannose *N*-glycans, which allow a weak but significant binding to DC-SIGN and L-SIGN, while sE2man strongly binds to both lectins. These results are in keeping with a recent paper (39)

showing that the presence of high-mannose *N*-glycans is the principal determinant of virus glycoprotein interaction with C-type lectins DC/L-SIGN. Structural analyses of the CRD of L-SIGN in complex with a Man9 glycan support this interpretation as they prove that only the outer trimannose branch point of the glycan in its unmodified high-mannose form binds to the CRD. The binding of the inner trimannose branch point to the CRD is blocked due to steric hindrance caused by a clash of Phe-325 of L-SIGN with a core *N*-acetyl-glucosamine of the glycan (34). Deglycosylation of sE2man with PNGase F under native conditions allowed us to partially remove the high-mannose *N*-glycans from sE2 indicating that the majority of the 10 E2 glycosylation sites are accessible at the surface of the soluble E2 molecule without denaturation. The deglycosylated sE2 preparation bound three times less to DC/L-SIGN than sE2man, but the residual high-mannose *N*-glycans still allowed significant binding compared with control. At present we do not know where these PNGase F-resistant *N*-glycans are located and whether they are part of a preferential binding site.

In our experiments high affinity binding correlates with close association of lectin CRDs, and we observe the highest affinity for sE2man when the CRD is part of a plasma membrane-expressed tetrameric lectin. This strongly suggests that multiple contacts are made between several high-mannose *N*-glycans on sE2man with the tetrameric CRD. In support of this model are results obtained in the HIV model, where high-mannose *N*-glycans were modeled on the structure of the trimeric envelope glycoprotein gp120. Results obtained by other groups suggest that DC-SIGN interacts with high-mannose *N*-glycans located on the outer surface of the envelope trimer in clusters facing the cell (39, 54). Biochemical data further support the view that up to four high-mannose *N*-glycans in such clusters might recognize the four CRD subunits of the lectin tetramer (33). Structural data are now needed to analyze the interaction of virus glycoprotein with lectin CRDs.

We speculate that DC-SIGN and L-SIGN might be molecular determinants for tissue tropism of HCV based on a growing body evidence. The virus is mainly transmitted by intravenous injection (drug use, contaminated needle sharing, blood transfusion) (55), and it is likely that HCV encounters circulating or tissue-resident dendritic cells (DC). In keeping with this assumption, replicating antigenomic HCV minus-strand RNA has been detected in DC *in vitro* (6) and more importantly *in vivo* (5, 56). Regarding the interaction with DC-SIGN it has been shown that this lectin facilitates virus entry into DC in *cis* by enhancing attachment of HIV (27), Ebola virus (28), cytomegalovirus (29), and Dengue virus[2] to the cell, thereby increasing the likelihood of interaction with specific entry receptors. Besides playing a role in entry into DC, HCV E2 interaction with DC-SIGN might also be detrimental for the interaction of DC with T cells during antigen presentation (35). Deficiencies in allogenic stimulation of T cells have indeed been observed in HCV-infected patients (56), but further studies using infectious HCV patient serum or E1/E2-pseudotyped viruses (53) will be needed to explore the functional aspects of HCV-DC-SIGN interaction.

Our results on E2 binding to L-SIGN might play an important role in receptor-mediated targeting of HCV to the liver. Indeed, the selective expression of L-SIGN in liver sinusoidal cells is in favor of this hypothesis. Following contamination by infected blood, HCV might bind to L-SIGN on sinusoidal endothelial cells resulting in concentration of virions in the liver. Productive infection of endothelial cells by HCV has not been demonstrated, but L-SIGN could be responsible for the transmission of bound virus to neighboring hepatocytes. This kind of mechanism of *trans*-enhancement has been demonstrated more than a decade ago for HIV transmission from DC to T cells (57) and can be attributed to DC-SIGN (27). Internalization of HIV might be important in this process as mutations of a dileucine motif in the cytoplasmic domain of DC-SIGN have been shown to abolish enhancement of HIV infectivity (58). In the case of HCV, it is tempting to speculate that subsequent to interaction with L-SIGN on endothelial cells, the virus could be transmitted to hepatocytes where it uses a specific receptor for entry. Candidates include CD81 (23), the scavenger receptor SR-BI (25), the LDL receptor (21, 22), or the asialoglycoprotein receptor (26), which is expressed on liver hepatocytes. Like DC-SIGN and L-SIGN, the asialoglycoprotein receptor attaches HCV through binding of glycan residues (galactose or *N*-acetyl-galactosamine) on the viral glycoprotein E2.

In conclusion, our results show that HCV envelope glycoprotein E2 strongly binds to oligomeric C-type lectins in a high-mannose *N*-glycan-dependent fashion. High affinity interaction of viral glycoproteins with lectins might represent a strategy by which enveloped viruses target to the site of replication and represents an interesting novel target for antiviral drug development.

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DC-SIGN and L-SIGN Are High Affinity Binding Receptors for Hepatitis C Virus Glycoprotein E2

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